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BLOTTING AND SEQUENCING TECHNIQUES
IMPORTANCE IN VETERINARY DERMATOLOGY

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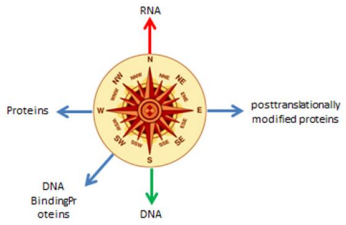
The goal of this second presentation is not to present molecular tools that are currently used in daily practice but more to introduce laboratory techniques that have shed or that will shed some new light on common dermatological diseases. It is not mandatory to know these tools to be good dermatologists but it is certainly necessary to understand these techniques to read and understand recently published research papers!

BLOTTING

Blotting is a powerful and sensitive technique for identifying the presence of specific biomolecules within a sample. The first of these techniques developed was the Southern blot, named for Dr. Southern, who developed it to detect specific DNA sequences. Subsequently, the method was modified to detect other targets. The nomenclature of these techniques was built around Dr. Southern's name, resulting in the terms northern blot (for detection of RNA), western blot (for detection of protein), eastern blot (for detection of posttranslationally modified proteins), and southwestern blot (for detection of DNA binding proteins). The last two techniques (eastern blot and the southwestern blot) are variations of western blots rather than distinct entities.

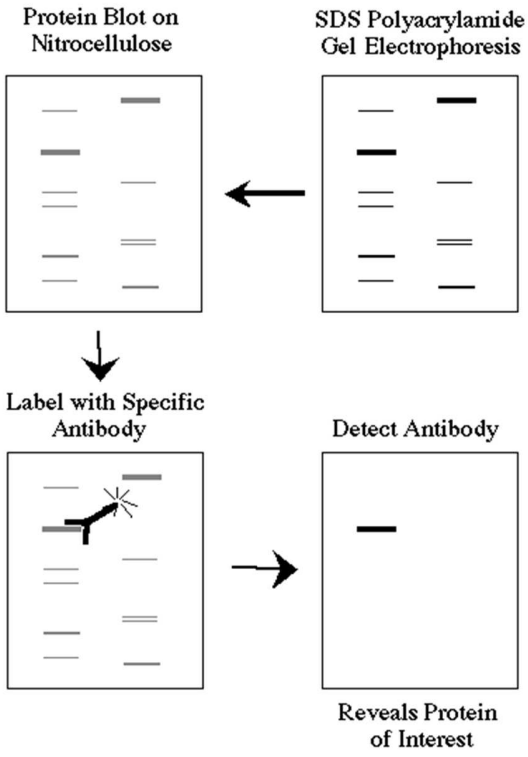


THE BLOTTING PROCESS

The principles of these techniques are identical:

1. The target molecules (DNA, RNA, or protein) are separated by a combination of their size and charge using the appropriate method of gel electrophoresis.
2. Separated molecules are transferred to a membrane
3. The membrane is treated with a probe directed against the specific molecule of interest.

In the first step, large target molecules may first be modified to facilitate movement through the gel. Very large sequences of DNA are processed with restriction endonucleases to cut them into smaller pieces. RNA is usually processed without any modification. Proteins may be denatured with specific detergents or heat. Denaturing a protein means unfolding it from its naturally occurring three-dimensional structure to a partially or completely linear structure. The denaturing step also disrupts most protein¯protein interactions. Most commonly, sodium dodecyl sulfate, a detergent, is used to denature proteins in this setting. It also provides a stronger negative charge, which facilitates gel electrophoresis.



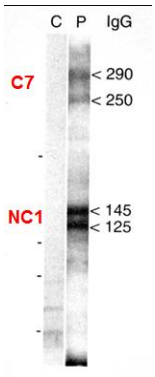
The sample is then loaded onto a gel where, in response to application of an electrical charge, molecules vertically separate based on size and charge, with smaller and more charged molecules running through the gel more rapidly and thus traveling farther than large molecules in the same period of time. A `ladder_ consisting of molecules of standardized and known sizes is run parallel with the samples, which allows the technician to estimate the size of each unknown molecule. In the second step, the molecules are horizontally transferred to a membrane, again using an electrical gradient, which maintains the vertical separation established by gel

electrophoresis. This step is akin to making a carbon copy, except the molecules themselves are transferred, rather than an image.
Finally, the membrane is treated with probes that bind only the specific molecule being sought. The type of probe varies based upon the target molecule. For nucleic acids (DNA and RNA), the probe is a labeled complementary (antisense) strand that hybridizes to the target sequence. For proteins, a monoclonal antibody (the primary antibody) binds to the protein of interest. This is followed by a second antibody (the secondary antibody), which recognizes the Fc portion of the primary antibody and is itself labeled, allowing specific detection. This `two-step_ method is used for cost control because labeling each specific monoclonal antibody would be quite expensive. Because of the use of antibodies, the western blot technique is sometimes referred to as immunoblotting. Regardless of which form of probe is used, the label may be a radioactive isotope or a fluorescent or chromogenic dye, all of which allow detection of the presence of the probe under specific conditions.

CURRENT APPLICATION of BLOTTING

As mentioned above, blotting aims at identifying specific biomolecules involved in a pathological process.
In the last years, western blotting was, in example, used for the identification of antigens involved in auto-immune dermatoses or for identification of allergens responsible of some allergic reactions. In these last two cases, western blotting was used because the goal of the study was the identification of proteins.

Epidermolysis bullosa acquisita

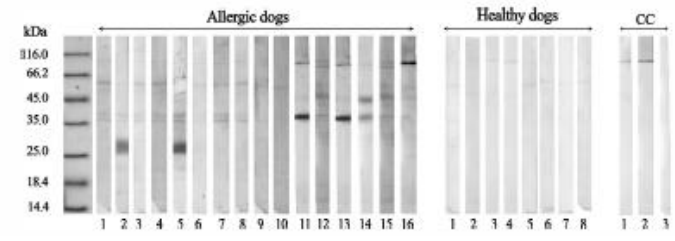


A few years ago, a clinical and histological counterpart of the human auto-immune dermatosis epidermolysis bullosa acquisita was described in dogs, mainly in great Danes. It was already known that this disease is associated in humans with antibodies directed against the NC1 part of collagen VII. To demonstrate that the canine disease is the exact counterpart of the human one, it was necessary to show that the same antigens are targeted by the immune reaction. Sera from the affected

dogs were then collected, as well as healthy dogs dermal samples. Proteins of the these dermis samples were separated using electrophoresis and then incubated with the sera of the affected animals. Sera of healthy individuals were used as controls. On the picture on the right, the membrane of the healthy individuals (marked C) does not exhibit any blotting, while the membrane of the affected dog (marked P) has sevral blotting bands. One of them has a molecular weight of 145 KDa, which is the expected weight of the NC1 part of collagen VII.

Ambrosia (ragweed)-induced atopic dermatitis

Canine atopic dermatitis is often associated with serum IgE directed against environemental allergens. This sensitization is usually tested with crude allergens extracts, which contain, basically, all proteins of this pollen, fungus or mite. Testing with these mixtures may lead to false-positive or false negative results in testing and identifying the protein(s) in these extracts that are really culprit is mandatory. The great majority of these allergens have been identified in man but very few is known on canine allergens. Moreover, it has been shown that house dust mites sensitized atopic dogs do not respond to the same house dust mites allergens that human counterpart. That is the reason why studies should be conducted to identify the canine allergens.



In the presented study atopic dogs with a known exacerbation during ambrosia pollinization season and positive skin tests for this pollen were included. Ser from these dogs were collected. Ragweed proteins were extracted and separated with electrophoresis. Membranes with these proteins were subsequently incubated with sera of the atopic dogs! Most of the included dogs (81%) got a positive blotting for a protein with a 38KDa molecular weight which was subsequently identified as Amb a1, the major allergen of ambrosia in humans.

Southern blotting was not used very often in veterinary dermatology research but may be helpful to determine the form of an infectious agent gene in the host cells, for example. This was tremendously important for the understanding of the role of high-risk

papillomavirus for the development of cervical carcinomas in women. It was first shown that most cervical cancer harbored sequences of papillomaviruses, especially HPV16. It was subsequently shown that the oncogenic potential of the virus is associated with the integration of a part of its genome within the genome of the host and the concomitant deletion of some regulatory genesŭ This major discovery was mainly made with southern blotting. Details and explanations will be given during the lectures.

GENOMICS, TRANSCRIPTOMICS: HOW THE SEQUENCING TECHNIQUES WILL (have already) CHANGE(d) DERMATOLOGY!

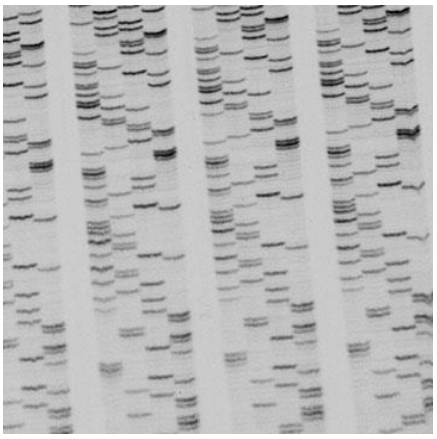
Nucleic acid sequencing is a method for determining the exact order of nucleotides present in a given DNA or RNA molecule. In the past decade, the use of nucleic acid sequencing has increased exponentially as the ability to sequence has become accessible to research and clinical labs all over the world. The first major foray into DNA sequencing was the Human Genome Project, a $3 billion,13-year-long endeavor, completed in 2003. The Human Genome Project was accomplished with first-generation sequencing, known as Sanger sequencing. Sanger sequencing (the chain-termination method), developed in 1975 by Edward Sanger, was considered the gold standard for nucleic acid sequencing for the subsequent two and a half decades.
Since completion of the first human genome sequence, demand for cheaper and faster sequencing methods has increased greatly. This demand has driven the development of second-generation sequencing methods, or next generation sequencing (NGS). NGS platforms perform massively parallel sequencing, during which millions of fragments of DNA from a single sample are sequenced in unison. Massively parallel sequencing technology facilitates high-throughput sequencing, which allows an entire genome to be sequenced in less than one day.

FROM SANGER SEQUENCING TO NGSŭ

The classical chain-termination method (the so-called Sanger method) requires a single-stranded DNA template, a DNA primer, a DNA polymerase, normal deoxynucleosidetriphosphates (dNTPs), and modified di-deoxynucleotidetriphosphates

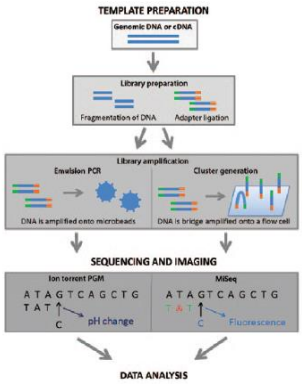
(ddNTPs), the latter of which terminate DNA strand elongation. These chain-terminating nucleotides lack a 3'-OH group required for the formation of a phosphodiester bond between two nucleotides, causing DNA polymerase to cease extension of DNA when a modified ddNTP is incorporated. The ddNTPs may be radioactively or fluorescently labeled for detection in automated sequencing machines.

The DNA sample is divided into four separate sequencing reactions, containing all four of the standard deoxynucleotides (dATP, dGTP, dCTP and dTTP) and the DNA polymerase. To each reaction is added only one of the four dideoxynucleotides (ddATP, ddGTP, ddCTP, or ddTTP), while three other nucleotides are ordinary ones. Putting it in a more sensible order, four separate reactions are needed in this process to test all four ddNTPs. Following rounds of template DNA extension from the bound primer, the DNA



fragments are heat denatured and separated by size using gel electrophoresis. This is frequently performed using a denaturing polyacrylamide-urea gel with each of the four reactions run in one of four individual lanes (lanes A, T, G, C). The DNA bands may then be visualized by autoradiography or UV light and the DNA sequence can be directly read off the X-ray film or gel image (See picture: each lane correspond to one nucleotide).

As already mentioned demand for cheaper and faster sequencing methods has increased greatly. This demand has driven the development of second-generation



sequencing methods, or next generation sequencing (NGS). Several NGS platform are now available but they are all based on the so called `sequencing by synthesis_ principle: The first step consists into construction of sequencing libraries. Basically, DNA samples are fragmented and smaller DNA sequences are ligated to adapter sequences. These new sequences are clonally amplified and subsequently act as templates, of which new DNA fragments are synthesized. As nucleotides are sequentially incorporated, they are digitally recorded as sequence (See illustration below, from Grada and al. 2013). Once sequencing is complete, raw sequence data must undergo several analysis steps. A generalized data analysis pipeline for NGS data includes preprocessing the data to remove adapter sequences and low-quality reads, mapping of the data to a reference genome or *de novo* alignment of the sequence reads, and analysis of the compiled sequence. Analysis of the sequence can include a wide variety of bioinformatics assessments, including genetic variant calling for detection of SNPs (single nucleotide polymorphisms) or indels (i.e., the insertion or deletion of bases), detection of novel genes or regulatory elements, and assessment of transcript expression levels. Analysis can also include identification of both somatic and germline mutation events that may contribute to the diagnosis of a disease or genetic condition.

FAM83G gene mutation in dogs with footpad hyperkeratosis

These new sequencing technologies have been used recently to identify the gene associated with footpad hyperkeratosis in dogs. Interestingly, the gene was identified initially with the inclusion of only 13 affected dogs from the breed Kromfohrländer and 29 control dogs. In a second step, the same mutation was identified in 23 affected Irish terriers. This study demonstrates that, at least for disease linked to one single gene, the available powerful techniques may be successfully used, even for rare diseases.

16s pyrosequencing: the microbiome studies

The pyrosequencing is probably the most used technology for the study of the human microbiome. One of the greatest advantages of the massive parallel pyrosequencing approach over the Sanger sequencing method is that hundreds of thousands of sequence reads can be obtained in a single run, generating sequence information data that are orders of magnitude larger. However, most of the currently used NGS platforms generate sequence reads that are too short in length. Because of the short sequence reads, bacterial identification using these methods has focused primarily on hypervariable regions of the 16S rRNA gene. It has been shown that reads spanning these particular regions of the 16S rRNA gene can still be highly informative and that despite the shorter read lengths, the pyrosequencing approach provides a description of the microbiome that is in good agreement with that provided by the cloning and Sanger sequencing approach in terms of higher taxonomic levels and relative abundance values.

Using this approach, several teams demonstrated the unbelievable diversity of the human skin microbiome: More than 4,700 unique taxa were, in example, detected across all of the hands examined! It was also possible to demonstrate that the microbiome of the head differs deeply from the arm region.

A few months ago, the first veterinary study on skin microbiome in atopic dogs has been published. Using pyrosequencing, the authors have demonstrated that 1) The skin microbiome of the dogs is as diverse as the human one 2) the microbome differs from one region to another and 3) the microbiome of atopic dogs is less rich in terms of species diversity when compared to healthy individuals.

We are currently using a similar technique to check whether the bitches are transmitted their skin flora to the puppies and whether this flora influence the development of atopic dermatitis in dogs.

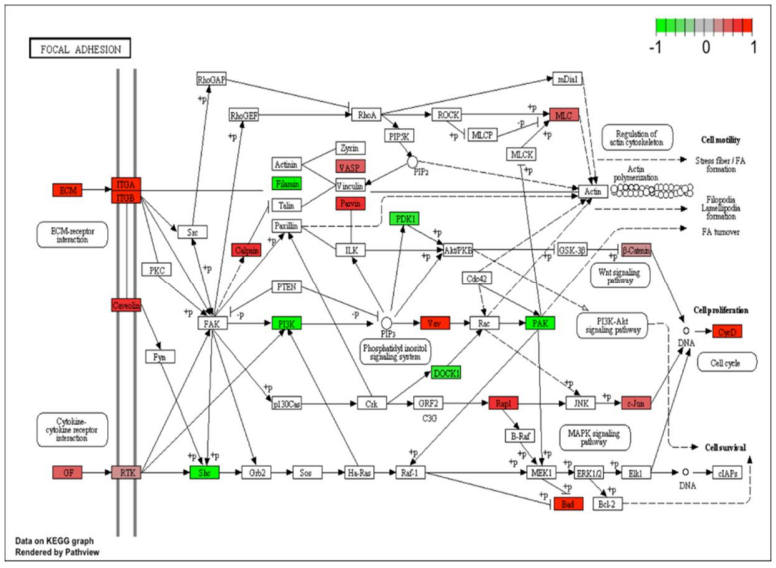
RNA seq and the transcriptomic studies

Sequencing techniques may also be applied to RNA. The transcriptome is defined as the complete set of transcripts in a cell, and their quantity, at a specific stage of the cells or tissue development. With this approach, not only the mRNAs but also the non-coding RNA and the small RNAs are detected. Splicing patterns and post-transcriptionnal

modifications may also be detected. Transcriptomes were first studied using microarrays. These so-called `expression chips_ relied on RNAs hybridization and the use of nucleic acids immobilized on a solid surface (the chip). These hybridization-based approaches are high throughput and relatively inexpensive but they have also several limitations. The main drawback is the reliance upon existing knowledge about genome sequences: In other words, one can only detect and measure the expression of already identified genes!

In contrast to microarray approach, sequence-based methods determine directly the cDNA sequences. The most popular of these methods, the so-called RNA seq, has clear advantages over existing approaches and is expected to revolutionize transcriptomics. This is mainly because this technique is not limited to detecting transcripts that correspond to existing genomics sequences and because of its high sensitivity and specificity.

We have recently used this approach to uncover markers of the development of PV-induced penis carcinomas in horses. In a first study, we have demonstrated that up to



20% of the healthy horses are carrier of EcPV2 (the putative agent of this cancer transformation) and that 40% have ECPV2-specific antibodies. As penis carcinomas are uncommon, we have decided to try to identify markers of this cancer transformation. We have consequently included 5 affected horses and three healthy individuals, extracted RNA from penis biopsies and run RNA seq analyses. Up to 15,000 genes shown to be up- or down-regulated, 2,000 of them with a very high level of significance. This approach demonstrated that some cellular pathways, such as cell growth and differentiation or cell adhesion, or biological processes were deeply affected. Last but not least, comparing our results with those of

similar studies allowed us to propose two genes, namely MMP1 and IL8 as potential markers.

In conclusion, researchers use on a daily basis, old (blotting) or new (sequencing) techniques to better determine the role of specific biomolecules (Proteins, DNA, RNA) in clinical processes. Understanding how these techniques work, their indications, advantages and drawbacks is mandatory for clinicians wanting to understand the present and future development of medicine.

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